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Isoelectric Focusing Patterns of Staphylococcal Exfoliative Toxin

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Abstract. Multiple components in two antigenic variants of staphylococcal exfoliative toxin were demonstrated by isoelectric focusing in polyacrylamide gel. The main components had an isoionic point of pH = 7.0 while other components ranged from pI = 4.5 to pI = 8.5. Each individual component was shown to possess full biologic and serologic activity after focusing. Isoelectric focusing in the presence of 8 M urea also showed microheterogeneity in exfoliative toxin preparations. The various components appear to be reversible conformers, possibly raised by exposure to the pH gradient of the ampholines.

Epidermal exfoliative disease has been shown to be caused by toxins elaborated by certain strains of *Staphylococcus aureus* [10]. We have previously reported the isolation of two such exoproteins from phage group II strains [8,9]. Both toxins were purified by the same procedure: concentration by ultrafiltration, chromatography on carboxymethyl cellulose followed by chromatography on hydroxylapatite. The toxins had similar molecular weights (26,000–27,000 daltons) and similar amino acid compositions. They showed significant homology of their primary structures in the amino terminal region and had identical biological activity. They were, however, serologically distinct, giving reactions of nonidentity in Ouchterlony immunodiffusion.

Although each protein was homogeneous by the usual physical and immunological methodologies, we observed an apparent heterogeneity in isoelectric focusing. A similar phenomenon has been noted in exfoliative toxins isolated from other strains of *S. aureus* [1]. In this report, we demonstrate that the various isoelectric components are reversible conformers that possess full biological activity. The transitions are ascribed to exposure to the pH gradient and/or ampholyte binding.

Materials and Methods

Purified exfoliative toxin samples were prepared as previously described from *S. aureus* strains TA and DI [8,9], hereafter referred to as ETA and ETB, respectively. Toxins were stored as lyophilized powders and reconstituted immediately before use. Due to a limited supply of highly purified ETB, the resegregation experiments and the focusing of urea-denatured toxin were performed with ETA only.

Polyacrylamide gels for electrofocusing were prepared from a stock solution of 30% acrylamide and 1.8% bis-acrylamide (Bio-Rad, Richmond, CA). The gel mixture contained 2.0 ml of the stock acrylamides, 10.0 ml distilled water, and 750 μl of the selected ampholines. When urea was incorporated, the water was reduced to 6.0 ml and 8.25 g of urea (Schwartz-Mann, Orangeburg, NJ) were added to the gel mixture. The mixture was degassed for 10–20 min, then 30 μl of 10% ammonium persulfate (freshly prepared) and 20 μl of TEMED (N,N,N′,N′-tetramethylethylenediamine) were added. Individual cylindrical gel tubes (5 mm internal diameter) were filled to a height of 9 cm. An overlay of distilled water was added and the gels were allowed to polymerize for 60 min.

Electrode solutions in the gel focusing chambers were 30 mM NaOH for the top electrode and 10 mM H_3PO_4 for the bottom electrode. Gels were prefocused for 1 h at 200 V. Samples were made dense by adding an equal volume of 25% sucrose, then layered through the top electrode solution onto the tops of gels. Focusing was continued at room temperature for 20 h at 500 V (1 mA per tube). At the completion of the run, the gels were removed from the tubes by rimming with water, then immersed in fixing solution containing 17.3 g sulfosalicylic acid and 57.5 trichloroacetic acid in a mixture of 150 ml methanol and 350 ml distilled water. After 1 h of fixation, the gels were stained with Coomassie brillant blue R250 (0.1%), dissolved in the destain solution, which contained 8% acetic acid in a mixture of water:ethanol (3:1).

In order to determine the pH gradient in the gel, a surface electrode (Ingold) was used to measure the pH at 1-cm intervals. Alternatively, 20 segments of 0.5 cm each were cut from an unstained gel. Each gel segment was then immersed in 100 μl distilled water for 24 h at 4°C and the pH determined on the eluate. Eluates were also obtained to test immunological and biological activity of each component; for these studies, slices were made to correspond with protein bands of an identical gel.

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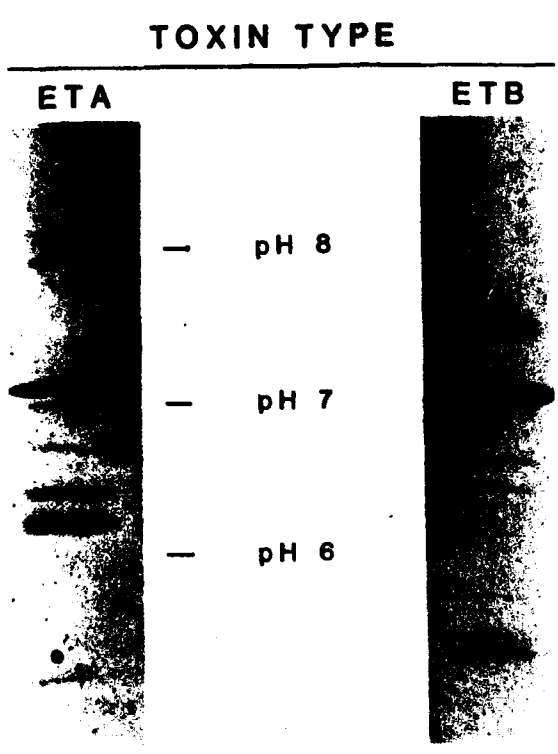

Fig. 1. Thin-layer isoelectric focusing patterns of purified staphylococcal exfoliative toxins (ETA and ETB) with superimposed pH gradient.

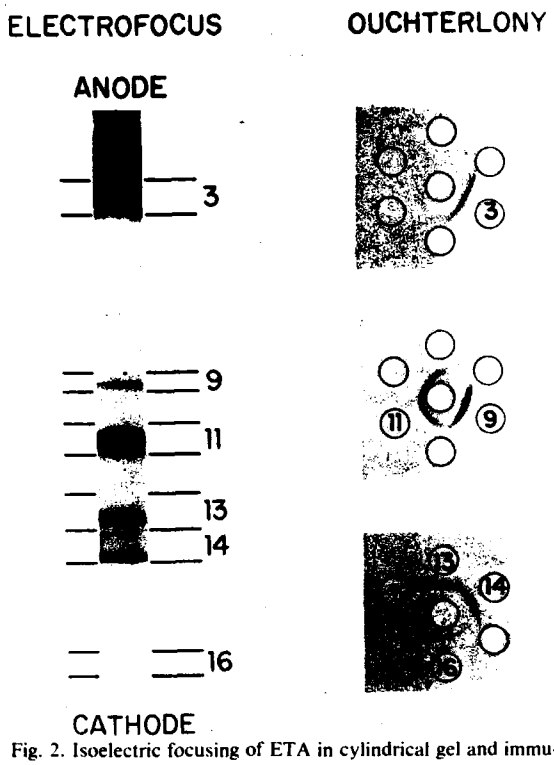

Fig. 2. Isoelectric focusing of ETA in cylindrical gel and immunodiffusion plate showing reactivity of individual slices. Slices were made to correspond with protein-stained bands. Center well contained antiserum to the purified toxin.

Commercially prepared thin-layer isoelectric focusing gels (PAG plates) with a pH gradient of 3.5–9.5 were supplied by LKB (Rockville, MD) and used according to the manufacturer's directions. Identical samples were applied at various positions on the gel, that is, near the cathode, near the anode, and near the center of the plate.

Biological activity of individual components was tested by subcutaneous injection into neonatal mice. Mice were observed and scored for a positive Nikolsky sign as previously described [2]. Immunological activity was determined by double diffusion in agar, using specific antiserum raised by injection of purified toxin into rabbits.

Results

Isoelectric focusing of exfoliative toxin. Focusing patterns from purified toxins (both ETA and ETB) showed marked heterogeneity (Fig. 1); the major component of both had an isoelectric point (pI) of 7.0–7.1. As many as 16 components were seen in both toxin preparations; some components had a pI higher than the major component (at about pH 8) while others focused at lower pH values. We examined this pattern of various isoelectrically different components by using ampholines produced by several manufacturers. The LKB ampholine and Pharmalyte (Pharmacia) gave essentially identical results, while patterns observed with Bio-Lytes (Bio-Rad) were similar but not as reproducible (data not shown). Increasing the time of focusing or the voltage applied to the gel did not alter the patterns of microheterogeneity. Virtually identical patterns were observed on thin-layer gels. No differences were seen when the samples were applied at different positions on the thin-layer gel. In addition, applying the sample after prefocusing the gel plate did not alter the pattern of microheterogeneity.

Toxin activity in individual components. By running samples of purified toxin (300 μg each) on duplicate cylindrical isoelectric focusing gels, we were able to stain one gel for protein bands, and slice the other gel to elute individual components. Eighteen segments of gel were analyzed for antigenic and toxic activity. Slices were made to correspond with segments of gel showing protein-stained material; that is, not all slices were made an arbitrary size, but

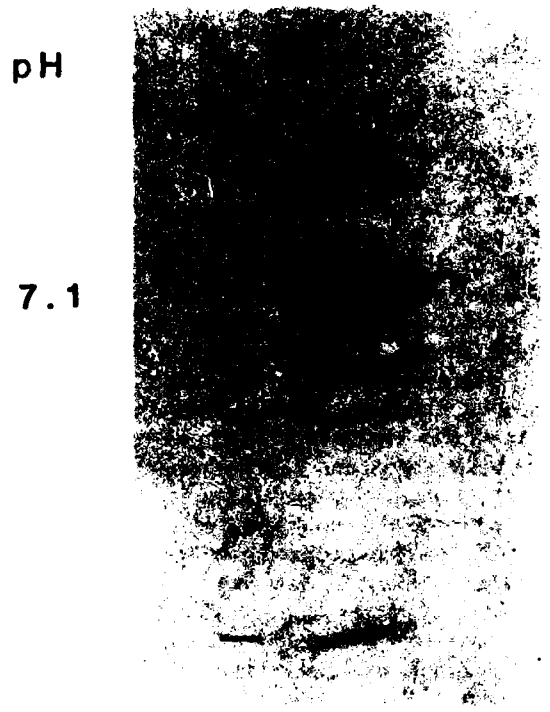

Fig. 3. Refocusing of ETA. The major component (pI = 7.0) was eluted from a cylindrical gel, then refocused on a thin-layer gel. Several protein bands are seen above and below the major component. The major component seen after refocusing had a pI = 7.1.

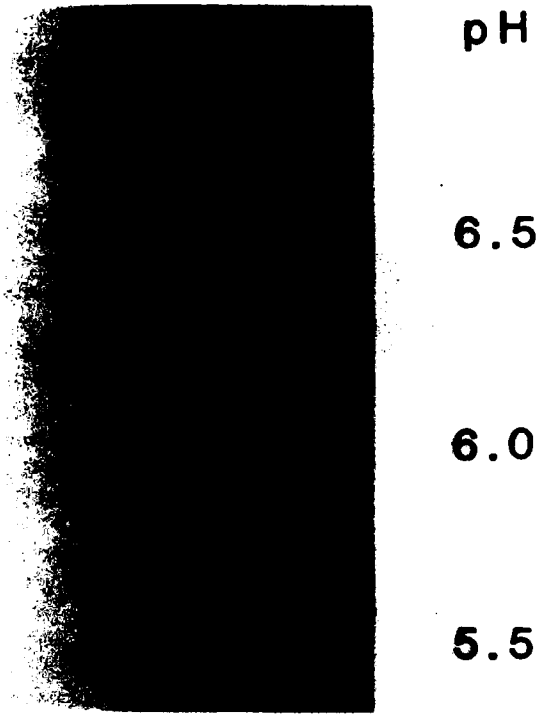

Fig. 4. Isoelectric focusing of ETA in the presence of 8 M urea. pH gradient is superimposed. Multiple protein bands are seen from pH 6.5 to 5.5.

rather were positioned to encompass only a single protein band. We found mouse toxicity in each slice corresponding to a stained protein band; the unstained area of the gel contained no toxic activity. We also demonstrated the antigenicity of each protein band (Fig. 2) by testing the eluates in gel diffusion with specific antiserum. These results indicate that the determinants for toxicity and antigenicity were not destroyed by the isoelectric focusing procedure, and further, that all components possessed the necessary amino acid sequences for antigenic and toxic specificity. The results shown in Fig. 2 were obtained with ETA. Similar results were seen with ETB.

Resegregation. Some of the major individual components of ETA were subjected to refocusing. After recovery from the gel slices, the major component (pI = 7.0), the pH 6.0 and the 8.0 species were refocused by application on a thin-layer gel with a pH range of 3.5–9.5. Both the pI = 7.0 and the pH 6.0 species demonstrated microheterogeneity (refocusing of pI = 7.0 component is shown in Fig. 3). Again, the main component had a pI = 7.0–7.1; in addition, at least four other components were demonstrated, three with lower pI and one with higher than the main component. These components all corresponded to ones seen in the focusing pattern of the original purified material. The pH 8.0 species showed a relatively strong band at pH 7.1. Due to the low concentration, no other bands were seen. The other minor components were not recovered in sufficient quantities to permit refocusing tests.

Effects of urea. Samples of purified toxin (ETA) were denatured in 8 M urea for 24–48 h at room temperature. Isoelectric focusing gels were cast containing 8 M urea and the standard ampholines at a concentration of 2%. Again the toxin showed a high degree of heterogeneity (Fig. 4); there were nine components easily visualized. The relative concentrations of the components showed a marked change from the non-denatured pattern in that no single component predominated. The pI range of

the individual components when focused in 8 M urea was less than 1 pH unit as contrasted with the non-denatured gels where the individual components were found over more than 4 pH units.

Discussion

The two exfoliative toxins demonstrated a high degree of heterogeneity in the patterns obtained in isoelectric focusing in polyacrylamide gels. Up to 16 bands were evident with isoionic points both above and below, as well as at, the isoelectric point of the bulk protein with a range from 4.5–8.5. Several explanations suggest themselves for the origin of this seeming paucidispersity. First, the preparations used may have been impure. Both physical and biological evidence argue against this. The toxins gave only a single line in SDS polyacrylamide gel electrophoresis and one peak on column chromatography [9]. They also were monodisperse and exhibited ideal behavior in the analytical ultracentrifuge.* Every band in the focusing gels whose concentration was high enough to test gave a serological reaction of identity in immunodiffusion and elicited a positive Nikolsky sign in neonatal mice.

A limited heterogeneity could also result from minor differences in covalent structure, for example, a substitution of one amino acid, or by a post-synthetic modification. This is precluded by the most critical experiment in these studies. Individual components were isolated from the gels from the isoelectric region and from both higher and lower pH regions. In each case, upon refocusing, the material was distributed in the original heterogeneous pattern. Deamidation, a well-documented cause of isoelectric heterogeneity [11,13], is also ruled out by this experiment. In addition, we have found that prolonged incubation of these toxins at pH 9.0 and 37°C did not modify the isoelectric focusing patterns (data not shown).

It appears likely that modification of the toxins during the course of the focusing is responsible for the apparent heterogeneity. The modification must be reversible and involve a conformational transition. It could be induced by ampholyte binding, by protonation on passage through the pH gradient, or by both of these mechanisms. Cann and co-workers have developed a phenomenological theory to explain multiple banding of a homogeneous protein in isoelectric focusing [4,7,14]. The theory considers both mechanisms but does not permit a choice between them. Our observation that carrier ampholytes from different manufacturers lead to identical focusing patterns suggests to us that pH-dependent conformational transitions are the more important factors for the exfoliatins. It seems less likely that the chemical composition and concentration of the many components in the several ampholyte mixtures are so similar as to generate isomerization to the same degree and with the same binding affinities. The ampholytes probably function primarily as strongly bound counter-ions.

The patterns obtained in concentrated urea are difficult to interpret. Although the pH range of the isoionic points was decreased and the relative concentrations were markedly altered, a considerable heterogeneity was still evident. Circular dichroism measurements (J.L. Middlebrook, unpublished observations) indicated that secondary structure was abolished in this solvent, but if the proteins were truly in random conformation, only one component would be anticipated. Perhaps ampholyte binding stabilized a family of partially unfolded species.

Isoelectric heterogeneity with homogeneous macromolecules is not unique to these two exfoliative toxins. Similar observations have been made with several proteins [6,12,15] and with transfer RNA [5] and in interaction with carrier ampholytes has been inferred in nearly all cases. The complexity of the pattern with the exfoliatins far exceeds that of any previously reported. It is noteworthy that there is a great deal of similarity in the patterns of the two molecules. This is suggestive of important conformational similarities, a not unlikely situation in view of their identical biological activities. Bailey et al. [3] reached the same conclusion based on the far ultraviolet circular dichroic spectra of their preparations of the presumed identical proteins (see footnote 1). They further suggested that an enhanced ellipticity in the near ultraviolet is indicative of an unusually rigid structure. However, in our laboratory the CD intensity of ETA is only one-half of theirs for tryptophan and less than one-sixth for tyrosine (J.L. Middlebrook, unpublished observations). We speculate that these differences may reflect a flexible structure readily permitting

* The molecular weights of these toxins were 27,030 for ETA and 26,530 for ETB for a partial specific volume of 0.73. These values are close to our previously reported molecular weights based on amino acid analysis and SDS gel electrophoresis [9]. Arbuthnott's laboratory [2] has reported molecular weights of 30,000 and 29,500, respectively, for immunologically identical materials, with very similar amino acid compositions. The bacterial strains of *S. aureus* from which the toxins were isolated were different, however, and it is not possible to make a definitive statement about whether the proteins are indeed the same.

the many conformational transitions described here.

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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